

APPLICATION FOR UNITED STATES LETTERS PATENT

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1004549.04609
"PDS" TO 2015

AN INTEGRATED SYSTEM FOR ANALYSIS OF BIOMOLECULES

This application is a continuation-in-part of pending provisional applications 60/262,530 and 60/262,852, both filed on January 17, 2001.

FIELD OF THE INVENTION

5 The present invention is related to the field of proteomics. More specifically, the present invention is a method and device for rapid identification and characterization of biomolecules recovered from biological media. Additionally, the present invention includes the ability to process numerous different samples simultaneously (high throughput analysis).

10 BACKGROUND OF THE INVENTION

Recent advances in human genome sequencing have propelled the biological sciences into several new and exciting arenas of investigation. One of these arenas, proteomics, is largely viewed as the next wave of concerted, worldwide biological research. Proteomics is the investigation of gene products (proteins), their various
15 different forms and interacting partners and the dynamics (time) of their regulation and processing. In short, proteomics is the study of proteins as they function in their native environment with the overall intention of gaining a further, if not complete, understanding of their biological function. Such studies are essential in understanding such things as the mechanisms behind genetic disorders or the influences of drug
20 mediated therapies, as well as potentially becoming the underlying foundation for further clinical and diagnostic analyses.

There are several challenges intrinsic to the analysis of proteins. First, and foremost, any protein considered relevant enough to be analyzed resides *in vivo* in a complex biological environment or media. Oftentimes, a protein of interest is present in
25 the media at relatively low levels and is essentially masked from analysis by a large abundance of other biomolecules, e.g., proteins, nucleic acids, carbohydrates, lipids and the like. Technologies currently employed in proteomics are only able to overcome this fundamental problem by first fractionating the entire biological media using the

old technology of two-dimensional (2D) sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), wherein numerous proteins are simultaneously migrated using a gel medium in two dimensions, i.e., as a function of isoelectric point and molecular size. In order to ensure migration in a predictable manner, the proteins are first
5 reduced and denatured, a process that destroys the overall structures of the proteins and voids their functionality.

Present day state-of-the-art proteomics involves the identification of the proteins separated using 2D-PAGE. In this process, gel spots containing separated proteins are excised from the gel medium and treated with a high-specificity enzyme (most commonly
10 trypsin) to fragment the proteins. The resulting fragments are then subjected to high-accuracy mass analysis using either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometries (MS). The resulting data, in the form of absolute molecular weights of the fragments, and knowledge of the enzyme specificity are used *in silico* to search genomic or protein databases for
15 information correlating to the empirical data on the fragments. Analytical methods and searching protocols, refined over the past seven years, have evolved to a point where only a few proteolytic fragments, determined with high mass accuracy, are needed to identify a gel-separated protein as being present in a certain gene.

However, identification of the gene responsible for producing a protein is only the
20 first step in the overall much larger process of determining complete protein structure and, finally, function. Numerous questions can be asked of protein structure/function that cannot be answered by the 2D-PAGE/MS approaches. One major issue deals with the primary structure of the protein. During the commonly practiced identification process, at most, fifty percent of the protein sequence is viewed, leaving at least fifty percent of the
25 protein unanalyzed. Given that potentially numerous splice variants, point mutations, and post-translational modifications exist for any given protein, many variants and modifications present within a protein will ultimately be missed during the identification process. As such, proteins are not viewed in the full structural detail needed to differentiate (normal) functional variants from (disease-causing) disfunctional variants.

Furthermore, current identification processes make no provision for protein quantitation. Because many disease states are created or indicated by elevated or decreased levels of specific proteins and/or their variants, protein quantitation is an essential component of proteomics. Presently, protein quantitation from gels is performed using staining approaches that inherently have a relatively high degree of variability, and thus inaccuracy. The staining approaches can be replaced using isotope-coded affinity tags (ICAT) in conjunction with mass spectrometric quantification of proteolytic fragments generated from 2D-PAGE. However, the ICAT approach is still subjective to the aforementioned protein variants, in that these structural variants will yield mass-shifted proteolytic fragments that will not be included in the quantification process. Likewise, other approaches, such as ELISA (enzyme-linked immunosorbant assay) and RIA (radioimmunoassay), are equally subjected to the complications of quantifying a specific protein (i.e., the functional variant) in the presence of its variants. Lacking the ability to resolve a target protein from its variants, these techniques will essentially monitor all protein variants as a single compound; a process that is oftentimes misleading in that a disease may be caused/indicated by elevated level of only a single variant, not the cumulative level of all the variants.

Moreover, the 2D-PAGE/MS and immunoassay approaches make no provision for exploring protein-ligand (e.g., other proteins, nucleic acids or compounds of biological relevance) interactions. Because denaturing conditions are used during protein 2D-PAGE separation, all protein-ligand interactions are disrupted, and thus are out of the realm of investigation using the identification approach. Alternatively, standard immunoassays, although performed under native conditions, do not include in the analysis a structural identification (i.e., an intrinsic physical property) component necessary for exact protein identification. Thus, other approaches are used in the analysis of protein-ligand interactions. The most frequently used of these are the yeast two-hybrid (Y2H) and phage display approaches, which use *in vivo* molecular recognition events to trigger the expression of genes that produce reporter proteins indicating a biomolecular interaction, or selectively amplify high-affinity binding partners, respectively. Other instrumental approaches rely on biosensors utilizing universal physical properties or tags

(e.g., surface plasmon resonance or fluorescence) as modes of detection. The two major limitations of these approaches is that they are generally slow and that interacting partners pulled from biological media are detected indirectly, yielding no specific or identifying information about the binding partner.

5 Lastly, none of the aforementioned approaches are favorable to large-scale, high-throughput analysis of specific proteins, their variants and their interacting partners in large populations of subjects. All of the aforementioned approaches require several hours (2D-PAGE) to several weeks (Y2H) to perform on a single sample. As such, time and monetary expenses preclude application to the hundreds-to-thousands of samples
10 (originating from hundreds-to-thousands of individuals) necessary in proteomic, clinical, and diagnostic applications.

To date, there are no universal, integrated systems capable of the high-throughput analysis of proteins for all of the aforementioned reasons. Thus, there exists a pressing need for new and novel technologies able to analyze native proteins present in their
15 natural environment. Encompassed in these technologies are: 1) the ability to selectively retrieve and concentrate specific proteins from biological media for subsequent high-performance analyses, 2) the ability to quantify targeted proteins, 3) the ability to recognize variants of targeted proteins (e.g., splice variants, point mutations and posttranslational modifications) and to elucidate their nature, 4) the capability to analyze
20 for, and identify, ligands interacting with targeted proteins, and, 5) the potential for high-throughput screening of large populations of samples using a single, economical platform.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the present invention
25 has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

SUMMARY OF INVENTION

It is an object of the present invention to provide an integrated system capable of selectively retrieving and concentrating specific biomolecules from biological media for subsequent high-performance analyses, quantifying targeted proteins, recognizing
5 variants of targeted biomolecules (e.g., splice variants, point mutations and post-translational modifications) and elucidating their nature, analyzing for, and identifying, ligands interacting with targeted biomolecules, and high-throughput screening of large populations of samples using a single, unified, economical, multiplexed and parallel processing platform.

10 It is another embodiment of the present invention to provide an integrated system that comprises molecular traps, such as affinity microcolumns, derivatized mass spectrometer targets, mass spectrometers capable of multi-sample input and robotics with processing/data analysis interactive database software that accomplish the high throughput analysis.

15 It is yet another object of the present invention to provide individual components for the integrated system, such as molecular traps, derivatized targets and the like.

It is a further object of the present invention to provide a high throughput embodiment of the present invention that uses robotics for serial preparation and parallel processing of a large number of samples simultaneously.

20 It is yet a further object of the present invention to provide methods and processes for use of the individual components and the integrated system in biological applications.

It is still yet another object of the present invention to provide a device and method for the identification of point mutations and variants of analytes using an integrated system using high throughput analysis.

25 It is yet another object of the present invention to provide an integrated system capable of quantifying specific functional variants of a protein while in the presence of dysfunctional, mass-shifted variants.

The novel features that are considered characteristic of the invention are set forth with particularity in the appended claims. The invention itself, however, both as to its
30 structure and its operation together with the additional objects and advantages thereof will

shown in Fig. 5b. The concentration range was spanned with good linearity ($R^2 = 0.999$) with overall standard deviation of the line of $< 2\%$.

With reference to Fig. 6, a bar analysis of the data shown in Fig. 4 using the standard curve constructed in Fig. 5 is illustrated. Each spectrum for the 88 samples from Fig. 4 was normalized to the equine β_2m signal through baseline integration, and the normalized integral for the human β_2m signal determined. Repetitive analyses were performed for each same individual and were averaged and the standard deviation calculated. The values of the averaged integrals were substituted in the equation derived from the standard curve and the concentration of human β_2m was calculated for each individual. The range of concentrations determined was from 0.75 to 1.25 mg/L. It is essential to note that by excluding mass-shifted variants (e.g., + 146 Da, due to glycosylated β_2m variant, which is dysfunctional), that these values indicate the true concentration of functional β_2m present in each individual, rather than the combined concentration of functional and dysfunctional variants.

Example 3: High Throughput Screening for Genetic and Posttranslational Variants

With reference to Fig. 7, a qualitative high-throughput screening of transthyretin (TTR) for posttranslational modification (PTM) and point mutations (PM) was performed using the integrated system and methods described herein. Aliquots of diluted (5-fold) human plasma samples collected from six individuals were prepared for parallel screening on a 96-well plate. Each well received a 15 μ L plasma aliquot (the samples from the six individuals were randomized on the 96-well plate), and 135 μ L of HBS buffer. Parallel sampling processing entailed simultaneous incubation/capture of the 96 samples on 96 anti-TTR derivatized microcolumns. The polyclonal anti-TTR microcolumns were made via glutaraldehyde-mediated coupling of the antibodies to amino-coated/modified microcolumns. Captured proteins were eluted from the microcolumn array with a small volume of MALDI matrix (saturated ACCA solution) and stamped onto a MALDI target array surface comprised of self-assembled monolayers (SAM) chemically masked to make hydrophilic/hydrophobic contrast targets. Each sample spot on the target array was analyzed using mass spectrometry and the relative TTR abundance determined by an automated MALDI-TOF mass spectrometric analysis

Fig. 2 is an illustration of a high-throughput semi-quantitative analysis of beta-2-microglobulin (β_2m) from human plasma samples using the integrated system and methods described in this invention.

Fig. 3 shows bar graph analysis of the data shown in **Fig. 2**.

5 **Fig. 4** is an illustration of a high-throughput quantitative analysis of β_2m from human plasma samples using the integrated system and methods described in this invention.

10 **Fig. 5a and 5b** illustrate the construction of a calibration curve from the data for the standard samples shown in **Fig. 4** and for the purpose of determining the β_2m concentrations in the human plasma samples screened via the high-throughput analysis using the integrated system and methods described in this invention.

Fig. 6 shows bar analysis of the data shown in **Fig. 4** using the standard curve constructed in **Fig. 5**.

15 **Fig. 7** is an illustration of a qualitative high-throughput screening of transthyretin (TTR) for posttranslational modification (PTM) and point mutations (PM) using the integrated system and methods described in this invention.

Fig. 8 illustrates identification of the posttranslational modifications and point mutations observed in the high-throughput TTR analysis using the integrated system and methods described in this invention.

20 **Fig. 9** illustrates the identification of point mutation via incorporation of derivatized mass spectrometer target platforms in the system and methods described in this invention.

25 **Fig. 10** illustrates the use of a high-resolution reflectron mass spectrometry as part of the integrated system and methods described in this invention in determining the identity of the point mutations detected in the analysis of the plasma samples shown in **Fig. 9**.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Example 1: An Integrated Mass Spectrometric Immunoassay System

1 The present invention provides an integrated system capable of selectively
retrieving and concentrating specific biomolecules from biological media for subsequent
high-performance analyses, quantifying targeted proteins, recognizing variants of targeted
biomolecules (e.g., splice variants, point mutations and post-translational modifications)
5 and elucidating their nature, analyzing for, and identifying, ligands interacting with
targeted biomolecules, and high-throughput screening of large populations of samples
using a single, unified, economical, multiplexed and parallel processing platform.

10 The preferred embodiment of the integrated system comprises molecular traps,
such as affinity microcolumns, derivatized mass spectrometer targets, mass spectrometers
capable of multi-sample input and robotics with processing/data analysis interactive
database. The present invention also includes methods and processes for use of the
individual components and the integrated system in biological applications. Furthermore,
the preferred embodiment of the present invention provides for the preparation and/or
processing of multiple separate devices and/or samples to accomplish high throughput
15 analysis.

20 A major component of the system of the present invention is the isolation or
retrieval of specific analytes from their surrounding biological media in a biological
sample. This is accomplished using a molecular trap. In a preferred embodiment of the
molecular trap, the retrieval process entails repetitively flowing the biological sample
through devices that have affinity receptors located on surfaces with a high surface area.
The affinity receptors are selected to capture specific analytes, and can be, e.g., proteins
(natural or recombinant), nucleic acids (DNA or RNA), antibodies, chelators, ion-
exchange moieties or low molecular weight organic or inorganic compounds such as
antibiotics, drugs or enzyme inhibitors. In the high throughput embodiment, these
25 molecular traps are formed into miniature columns, affinity microcolumns, thereby
allowing numerous molecular traps to be located side-by-side and taking up minimal
amount of physical volume.

30 The molecular trapping process is accomplished by allowing sufficient physical
contact between the affinity receptors located on the molecular traps and the analyte
contained in the biological sample. The affinity receptors capture, or isolate, the specific

analytes using an affinity interaction between the affinity receptors and the specific analytes. After the specific analytes are captured, residual or non-captured compounds are washed free of the molecular traps using a series of rinses. The capture and rinse processes result in the concentrating of the specific analytes into a low volume of the affinity microcolumns.

After the specific analytes have been captured, they are eluted from the molecular traps using a small volume of a buffer capable of disrupting the affinity interaction. The eluted specific analytes are then stamped directly onto a mass spectrometry target platform for either mass spectrometry or for further processing, e.g., enzymatic/chemical modification via utilization of bioreactive MS target arrays, followed by subsequent preparation for mass spectrometry. Automated mass spectrometry, preferably matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) in conjunction with time-of-flight, quadrupole ion-trap, Fourier transform ion cyclotron resonance (FT-ICR), magnetic/electric sector, or combinations thereof, mass analyzers, then follows with either the specific analyte or modified fragments detected with high precision. Software capable of recognizing differences between samples, or from a standard, is used to aid in the analysis and organization into database of the large numbers of samples.

The high throughput embodiment of the present invention uses robotics for serial preparation and parallel processing of a large number of samples. The use of microcolumns in capturing the specific analytes enables an arrayed format, as mentioned above, that is ideal for such high-throughput processing since it minimizes the physical volume occupied by the microcolumn array. Use of affinity microcolumns with appropriately configured robotics allows multiple samples to be prepared, processed, start-to-finish, simultaneously on a unified platform thereby enabling high throughput of samples. Specifically, all capture, separation and elution steps are performed within the microcolumns managed by the robotics system. This is in contrast to the use of other affinity capture methods (using, e.g., beaded media) where mechanical/physical means (e.g., centrifugation, magnetic or vacuum separation) are used to separate the specific analyte from the biological fluid and rinse buffers. Oftentimes this physical separation

needs to be performed singularly, resulting in the disruption of a parallel processing sequence, as well as the ordering of the array. Because these mechanical/physical means are not necessary when using the microcolumns, parallel-processing sequences can be used without disruption and the integrity of an ordered spatial array is maintained throughout the entire process. Most conveniently, multiple preparations/analyses are performed serially and in parallel using robotics fitted to commonly used spatial arrays, e.g., 4-, 16-, 48-, 96-, 384- or 1536-well micro-titer plate formats.

With reference to Fig. 1a the integrated system for high-throughput analysis of biomolecules from biological media comprises a prestation wherein pre-analysis processing, such as preparation of the array of molecular traps, is accomplished. From the prestation, the array of molecular traps are accessed by an initiation/reservoir/sample station or relocated to a use station for sample processing.

The initiation/reservoir/sample station is where sample media is located. Preferably, multiple samples are loaded into the initiation/reservoir/sample station and spatially arranged in an array commensurate with the array of molecular trap with one sample for each molecular trap in the array of molecular traps.

From the initiation/reservoir/sample station, the array of samples is automatically relocated to a use station. The use station is where the sample, and specific analytes contained therein are processed. In one embodiment, one end of the array of molecular traps is lowered into the sample and sample is drawn into each molecular trap. Since each molecular trap has affinity reagent located on surfaces of the molecular trap, drawing the sample into the molecular trap contacts any specific analyte sought after with the affinity reagent. Each molecular trap may have different affinity reagents from that of other molecular traps, thereby enabling the targeting of different specific analytes from the same or different media. By drawing sample into the molecular traps, specific analyte is placed into contact with the affinity reagent, which, through the affinity interaction, effectively captures the specific analyte. Sample material may be drawn into the molecular trap singly or multiple times. After sufficient specific analyte has been captured by the molecular trap, residual, non-captured, media are washed away with at least one rinse. (However, other embodiments may not require a rinse step.) After non-

targeted compounds have been washed away when desired, captured specific analyte are eluted from the molecular traps by contacting them with a solution selected to interrupt the affinity interaction. Finally, the eluted specific analytes are relocated to a target array by stamping them onto the target array.

5 The target array is then automatically relocated to a storing/loading station that is capable of containing at least one target array. From the storing/loading station, the target array is loaded into an automated mass spectrometer capable of multi-sample input and automatic processing/data analysis using interactive database.

10 Additionally, there may be a post-station for sample processing or additional analysis subsequent to mass spectrometric analysis.

15 With reference to Fig. 1b, a preferred embodiment of the use station further comprises a microcolumn manifold to which the array of molecular traps is attached. The microcolumn manifold attaches to a robotic head that is able to mechanically draw and expel biological media, rinses and MS matrices into/out of the attached microcolumn array. The manifold/robotic head combination is able to physically move the array of molecular traps between each processing station to accomplish a selected task, e.g, affinity isolation from biological media, rinse of extraneous compounds and deposition of extracted biomolecules for MS analysis. This physical movement of the microcolumn manifold may be in a rectangular (xy) or circular (carousel) manner. Alternatively, the microcolumn manifold in the use station may be stationary and the processing stations relocated under the array of molecular traps. Similar to the physical movement of the microcolumn array described above, the physical movement of the processing stations may be in a rectangular (xy) or circular (carousel) manner. Additionally, the microcolumn manifold/robotic head is capable of z-axis movement to facilitate motion over objects (e.g., titer plates, rinse basins and MS targets) present on the processing station.

25 An example using the integrated system is described below.

30 With reference to Fig. 2, a high-throughput semi-quantitative analysis of beta-2-microglobulin (β_2m) from human plasma samples using the integrated system and methods herein was performed. Aliquots of diluted (5 fold) human plasma samples collected from six individuals were prepared for parallel screening on a 96-well sample

plate. Each well received a 15- μ L plasma aliquot (the samples from the six individuals were randomized on the 96-well plate), 7.5 μ L of equine plasma (undiluted, containing equine β_2 m, $MW_{eq.\beta_2m}=11,396.6$, $MW_{hum.\beta_2m}=11,729.2$) and 128 μ L of HBS (0.01 HEPES, pH 7.4, 0.15 M NaCl, 0.005% (v/v) polysorbate 20, 3 mM EDTA) buffer. Eight of the 96 samples were chosen at random and 0.5 μ L of 10^{-2} mg/mL solution of β_2 m was added to four of them and 1 μ L of the same β_2 m solution to the other four wells. Parallel sample processing entailed simultaneous incubation/capture of the 96 samples on 96 anti- β_2 m derivitized microcolumns. The polyclonal anti- β_2 m microcolumns were made via carboxymethyl dextran (CMD)-EDC mediated coupling of the antibody to amino-coated/modified microcolumns. Captured proteins were eluted from the microcolumns with a small volume of MALDI matrix (saturated aqueous solution of α -cyano-4-hydroxycinnamic acid (ACCA), in 33% (v/v) acetonitrile, 0.2 % (v/v) trifluoroacetic acid) and stamped onto a MALDI target array surface comprised of self-assembled monolayers (SAM) chemically masked to make hydrophilic/hydrophobic contrast target arrays. Each sample spot on the target array was analyzed using mass spectrometry and the relative β_2 m abundance determined by an automated MALDI-TOF mass spectrometric analysis software routine. The mass spectra resulting from the high-throughput analysis of the 96 samples are shown in Fig. 2. Spectra taken from the samples that had the β_2 m standard solution added are shaded.

With reference to Fig. 3, which is a bar graph analysis of the data shown in Fig. 2, each spectrum shown in Fig. 2 was normalized to the equine β_2 m signal through baseline integration, and the normalized integral for the human β_2 m signal determined. All β_2 m integrals from spectra obtained from samples from the same individual were averaged and the standard deviation calculated. In the same way, the integrals for the samples spiked with 0.5 and 1.0 μ L solution of 10^{-2} mg/mL β_2 m were calculated and averaged. Plotted in this figure are the average values of the normalized human β_2 m integrals for the samples from the six individuals and the spiked samples. The bar graph clearly establishes increased β_2 m levels in the spiked samples, illustrating the value of the high-throughput semi-quantitative analysis performed with the system and methods described

in this invention in establishing increased $\beta_2\text{m}$ levels in human blood that are associated with various disease states.

Example 2: High Throughput Protein Quantification in the Presence of Variants

With reference to Fig. 4, a high-throughput quantitative analysis of $\beta_2\text{m}$ from human plasma samples was performed using the integrated system and methods described herein. The samples from six individuals were prepared as described in Fig. 2. Eighty-eight wells of the 96-well sample plate received 15 μL plasma aliquots (the samples from the six individuals were randomized on the 96-well plate), 7.5 μL of equine plasma (undiluted) and 128 μL of HBS buffer. A series of dilutions of a 7.6×10^{-4} mg/mL standard solution of purified human $\beta_2\text{m}$ were prepared (spanning a concentration range of 7.6×10^{-4} to 1.14×10^{-4} mg/mL) and used as samples (15 μL of each) in the last column (8 wells) on the 96-well plate. Parallel sampling processing and MALDI-TOF MS analysis was performed as described for Fig. 2, using the polyclonal anti- $\beta_2\text{m}$ microcolumns. The mass spectra resulting from the high-throughput analysis of the 88 samples and the 8 standards are shown in this figure. Spectra taken from the standard samples are shaded.

With reference to Fig. 5a and 5b, a calibration curve is constructed from the data for the standard samples shown in Fig. 4. The calibration curve is for the purpose of determining the $\beta_2\text{m}$ concentrations in the human plasma samples screened via the high-throughput analysis using the integrated system and methods described herein. Representative spectra of the data for each standard used to generate the working curve are shown in Fig. 5a. Each spectrum was normalized to the equine $\beta_2\text{m}$ signal through baseline integration over the m/z range of 11,390 – 11,410 Da, and normalized integrals for the human $\beta_2\text{m}$ signals (m/z range = 11,720 – 11,740 Da) determined. Integrals from five spectra taken for each calibration standard were averaged and the standard deviation calculated. A calibration (standard) curve was constructed by plotting the average of the normalized integrals for each standard vs. the human $\beta_2\text{m}$ concentration in the standard sample (adjusted for the human plasma dilution factor). The working curve generated is shown in Fig. 5b. The concentration range was spanned with good linearity ($R^2 = 0.999$) with overall standard deviation of the line of $< 2\%$.

With reference to Fig. 6, a bar analysis of the data shown in Fig. 4 using the standard curve constructed in Fig. 5 is illustrated. Each spectrum for the 88 samples from Fig. 4 was normalized to the equine β_2 m signal through baseline integration, and the normalized integral for the human β_2 m signal determined. Repetitive analyses were performed for each same individual and were averaged and the standard deviation calculated. The values of the averaged integrals were substituted in the equation derived from the standard curve and the concentration of human β_2 m was calculated for each individual. The range of concentrations determined was from 0.75 to 1.25 mg/L. It is essential to note that by excluding mass-shifted variants (e.g., + 146 Da, due to glycosylated b2m variant, which is dysfunctional), that these values indicate the true concentration of functional b2m present in each individual, rather than the combined concentration of functional and dysfunctional variants.

Example 3: High Throughput Screening for Genetic and Posttranslational Variants

With reference to Fig. 7, a qualitative high-throughput screening of transthyretin (TTR) for posttranslational modification (PTM) and point mutations (PM) was performed using the integrated system and methods described herein. Aliquots of diluted (5-fold) human plasma samples collected from six individuals were prepared for parallel screening on a 96-well plate. Each well received a 15 μ L plasma aliquot (the samples from the six individuals were randomized on the 96-well plate), and 135 μ L of HBS buffer. Parallel sampling processing entailed simultaneous incubation/capture of the 96 samples on 96 anti-TTR derivatized microcolumns. The polyclonal anti-TTR microcolumns were made via glutaraldehyde-mediated coupling of the antibodies to amino-coated/modified microcolumns. Captured proteins were eluted from the microcolumn array with a small volume of MALDI matrix (saturated ACCA solution) and stamped onto a MALDI target array surface comprised of self-assembled monolayers (SAM) chemically masked to make hydrophilic/hydrophobic contrast targets. Each sample spot on the target array was analyzed using mass spectrometry and the relative TTR abundance determined by an automated MALDI-TOF mass spectrometric analysis software routine. The mass spectra resulting from the high-throughput analysis of the 96 samples are shown in Fig. 7. In all of the spectra, the TTR signal is accompanied by

another signal at higher mass, indicating posttranslationally processed TTR form. In addition, all spectra resulting from the analysis of one plasma sample showed two additional signals at masses ~30 Da higher than the two “original” TTR signal.

With reference to Fig. 8, identification of the posttranslational modifications and point mutations observed in the high-throughput TTR analysis was performed using the integrated system and methods described herein. Shown are representative spectra resulting from analysis of samples from two individuals, showing the existence of two and four TTR signals, respectively. In the upper spectrum, two signals attributable to TTR are observed. The signals correspond well to the theoretically calculated mass of TTR ($MW_{TTR}=13,762$) and that of an oxidized TTR variant (TTR_{ox}) resulting from cysteinylolation at Cys10 (introducing a mass shift of +119 Da). In the lower spectrum, in addition to the above-mentioned two TTR signals, two additional peaks at masses ~ 30 Da higher than the two “original” TTR signal are observed. The identity of the point mutation is described in the next Example.

Example 4: High Throughput Identification of Point Mutations

With reference to Fig. 9, point mutations were identified by incorporating protease-derivatized mass spectrometer target array platforms into the system and using the methods described herein. The samples used were the same ones utilized for Fig. 8. TTR from diluted (50-fold, in HBS) human plasma was captured via polyclonal anti-TTR microcolumns, as described in Fig. 7. Instead of matrix elution, the captured proteins were eluted with a small volume of 10 mM HCl onto trypsin-conjugated targets containing buffered target spots (50 mM TRIS buffer pH 9.5) for sample pH modulation (buffer exchange). Shown in this figure are mass spectra resulting from a twenty-minute trypsin digest done at 40 °C of the proteins eluted from the anti-TTR microcolumns. The resulting two tryptic peptide maps localize the mutation in the tryptic fragment-12 (T_{12}), containing residues 104-127. A database search points to two possible TTR mutations in this region of the sequence: Ala109→Thr [DNA base change GCC→ACC], $\Delta m = 30.011$ Da, and Thr119→Met [DNA base change ACG→ATG], $\Delta m = 29.992$ Da. The identification of the correct mutation is shown in Fig. 10.

With reference to Fig. 10, high-resolution reflectron mass spectrometry forms a part of the integrated system and methods described herein in determining the identity of the point mutations detected in the analysis of the plasma samples shown in Fig. 9. The monoisotopic signal for the tryptic digest fragment T₁₂ (104-127) in normal (native) TTR shows at m/z = 2644.922, denoting Δm = 29.988 Da difference with the monoisotopic signal for the mutant TTR. Accordingly, the point mutations is assigned to Thr119→Met, Δm = 29.992 Da. This TTR point mutation results in a so-called “Chicago prealbumin” variant, a non-amyloid mutation. The results shown in Figs. 7, 8, 9, and 10 in combination illustrate the use of the system and the methods described herein in identifying posttranslational modifications and point mutations via concerted high-throughput screening analyses of biological samples.

The novel features that are considered characteristic of the invention are set forth with particularity in the appended claims. The invention itself, however, both as to its structure and its operation together with the additional objects and advantages thereof will best be understood from the following description of the preferred embodiment of the present invention when read in conjunction with the accompanying drawings. Unless specifically noted, it is intended that the words and phrases in the specification and claims be given the ordinary and accustomed meaning to those of ordinary skill in the applicable art or arts. If any other meaning is intended, the specification will specifically state that a special meaning is being applied to a word or phrase. Likewise, the use of the words “function” or “means” in the Description of Preferred Embodiments is not intended to indicate a desire to invoke the special provision of 35 U.S.C. §112, paragraph 6 to define the invention. To the contrary, if the provisions of 35 U.S.C. §112, paragraph 6, are sought to be invoked to define the invention(s), the claims will specifically state the phrases “means for” or “step for” and a function, without also reciting in such phrases any structure, material, or act in support of the function. Even when the claims recite a “means for” or “step for” performing a function, if they also recite any structure, material or acts in support of that means of step, then the intention is not to invoke the provisions of 35 U.S.C. §112, paragraph 6. Moreover, even if the provisions of 35 U.S.C. §112, paragraph 6, are invoked to define the inventions, it is intended that the inventions not be

limited only to the specific structure, material or acts that are described in the preferred embodiments, but in addition, include any and all structures, materials or acts that perform the claimed function, along with any and all known or later-developed equivalent structures, materials or acts for performing the claimed function.